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| (51) International Patent Classification ⁶ : A61K 47/48 // C07K 19/00 | A1 | (11) International Publication Number: WO 97/22364 (43) International Publication Date: 26 June 1997 (26.06.97) |
| <p>(21) International Application Number: PCT/IL96/00181</p> <p>(22) International Filing Date: 18 December 1996 (18.12.96)</p> <p>(30) Priority Data: 116436 18 December 1995 (18.12.95) IL</p> <p>(71) Applicant (for all designated States except US): YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM (IL/IL); 46 Jabotinsky Street, 91042 Jerusalem (IL).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): FISHMAN, Ala (IL/IL); Medical School of Hadasa, Dept. of cellular biochemistry, Ein Karem, 91120 Jerusalem (IL). YARKONI, Shai (IL/IL); Medical School of Hadasa, Dept. of cellular biochemistry, Ein Karem, 91120 Jerusalem (IL). LORBERBOUM-GALSKI, Haya (IL/IL); Medical School of Hadasa, Dept. of cellular biochemistry, Ein Karem, 91120 Jerusalem (IL).</p> <p>(74) Agent: NOAM, Meir; P.O. Box 32081, 91320 Jerusalem (IL).</p> | | <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p> |
| <p>(54) Title: FCϵ-PE CHIMERIC PROTEIN FOR TARGETED TREATMENT OF ALLERGY RESPONSES, A METHOD FOR ITS PRODUCTION AND PHARMACEUTICAL COMPOSITIONS CONTAINING THE SAME</p> | | |
| <p>(57) Abstract</p> <p>The present invention generally relates to a new approach for the therapy of allergic responses, based on targeted elimination of cells expressing the FcϵR1 receptor by a chimeric cytotoxin Fcϵ2-3-PEϵ40. A sequence encoding amino acids 301-437 of the Fc region of the mouse IgE molecule was genetically fused to PEϵ40 - a truncated form of PE lacking the cell binding domain. The chimeric protein, produced in <i>E. coli</i>, specifically and efficiently kills mouse mast cell lines expressing the FcϵR1 receptor, as well as primary mast cells derived from bone marrow. The present invention provides a chimeric protein for targeted elimination of FcϵR1 expressing cells especially useful for the therapy of allergic responses. The said chimeric protein is comprised of a cell targeting moiety for FcϵR1 expressing cells and a cell killing moiety. The preferred killing moiety is the bacterial toxin <i>Pseudomonas</i> exotoxin (PE). This <i>Pseudomonas</i> exotoxin is a product of <i>Pseudomonas aeruginosa</i>. The present invention also relates to a method for the preparation of said protein. This chimeric protein is prepared by genetically fusing the Fc region of the mouse IgE molecule to PEϵ40, a truncated form of PE lacking the cell binding domain. The present invention also provides pharmaceutical compositions, for the treatment of allergic diseases and for the treatment of hyperplasias and malignancies, comprising as an active ingredient the above mentioned chimeric protein and a conventional adjuvant product.</p> | | |

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Fcε-PE CHIMERIC PROTEIN FOR TARGETED TREATMENT OF ALLERGY
RESPONSES A METHOD FOR ITS PRODUCTION AND PHARMACEUTICAL
COMPOSITIONS CONTAINING THE SAME

FIELD OF THE INVENTION

The present invention generally relates to a novel approach for the therapy of allergic responses. More specifically the present invention relates to Fcε-PE chimeric protein for targeted elimination of FcεRI expressing cells, a method for its production, and pharmaceutical compositions containing the same. This chimeric protein is composed of cell targeting which is a part of IgE molecule linked to cell killing moieties for recognizing and destroying cells overexpressing the specific receptor. The killing moiety used in the chimeric protein of the present invention is the bacterial toxin *Pseudomonas* exotoxin (PE) (a product of *Pseudomonas aeruginosa*).

BACKGROUND OF THE INVENTION

About twenty percent of the world population suffers from various allergic diseases such as asthma, allergic rhinitis, food allergies, atopic dermatitis and anaphylaxis. The alarming increase in the prevalence of allergic diseases over the past decade has led to a clear need for more effective treatment.

The interaction between IgE and mast cells or basophils is the primary effector pathway in allergic responses. IgE binds to high-affinity receptor (FcεRI) for its constant region, found almost exclusively on the surface of these cells. The binding itself, in spite of the low dissociation rate, does not result in stimulation of the cell. However, cross-linkage of cell surface-bound IgE by multivalent antigen causes receptor aggregation, triggering explosive cellular degranulation whereby mediators of allergy such as

cellular degranulation whereby mediators of allergy such as histamine and serotonin are released.

The fact that distribution of the Fc ϵ RI receptor is restricted to cells participating in an allergic response makes it an attractive candidate for targeted immunotherapy by chimeric cytotoxins. Chimeric cytotoxins are a novel class of targeted molecules constructed by gene fusion techniques. These molecules are composed of cell targeting and cell killing moieties, enabling them to recognize and destroy cells overexpressing specific receptors.

The bacterial toxin *Pseudomonas* exotoxin (PE) used in chimeric protein constructs, is a product of *Pseudomonas aeruginosa*. Having accessed the cytoplasm, PE inhibits protein synthesis by its ADP-ribosylation activity, thus causing cell death (Middlebrook, J.I., and Dorland, R.B. 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. 48, 199.). Effective chimeric cytotoxins have been constructed by fusion of cDNAs encoding various growth factors or single chain antibodies with PE derivatives lacking intrinsic cell binding capacity. One of these chimeric proteins designated IL₂-PE₄₀, constructed to target and selectively eliminate activated T cells overexpressing IL₂ receptors, was shown to provide effective and selective immunosuppression in various models of autoimmune disorders, graft rejection and cancer (Lorberboum-Galski, H. 1994. Interleukin 2-Pseudomonas exotoxin A (IL2-PE40) chimeric protein for targeted immunotherapy and the study of immune responses. J. Toxicol.-Toxin Reviews, 13 (1), 105.).

The entire recombinant constant region of IgE (Fc ϵ) expressed in bacteria, have an affinity for Fc ϵ RI receptor comparable to that of the native IgE, as well as the capacity to sensitize basophils for anti-IgE induced histamine release. When recombinant fragments of human Fc ϵ expressed in bacteria, were tested for receptor binding, a peptide corresponding to residues 301-376 at the junctions

of domains 2 and 3 of the constant region was found to be sufficient for high-affinity binding to the receptor. It was also reported that ϵ -chain dimerization was not required for receptor binding (Helm, B., Marsch, P., Vercelli, D., Padlan, E., Gould, H., and Geha, R. 1988. The mast cell binding site on human immunoglobulin E. *Nature* 331, 180.).

The present invention generally relates to a novel approach for the therapy of allergic responses. At present the major known groups of drugs used in the treatment of asthma and allergic disorders are:

1. β_2 agonists - produce airway dilatation through stimulation of β_2 adrenergic receptors.
2. Methylxantines - smooth muscle relaxants, produce bronchodilatation.
3. Glucocorticoids - reduce inflammation.
4. Cromolyn sodium - prevents mast cell degranulation.
5. Antihistamines - prevents histamine action on its target cells.

Although widely used, all of these drugs have notable disadvantages in regard to:

1. Specificity: The action of all of these drugs (except cromolyn sodium) is not mast cell specific. Therefore, they can not prevent the release of allergy mediators but rather reverse or block the effects caused by their action. The treatment by these drugs is symptomatic, it can be started only after the onset of the allergic reaction and thus can't be used in a prophylactic manner.

2. Toxicity: Being non-specific, these drugs exert their action on various tissues and organs causing serious side effects. The major side effect of β_2 agonists is tremor, but they also cause cardiac arrhythmias; Methylxantines stimulate the central nervous system, causing nervousness, nausea, vomiting, anorexia, headache and cardiac muscle-causing tachycardia. At high plasma levels there is a danger of seizures and arrhythmias. Antihistamines affect the central nervous system, causing sedation. Steroids are most harmful, causing suppression of the pituitary-adrenal function, fluid and electrolyte disturbances, hypertension, hyperglycemia, increased susceptibility to infections, osteoporosis and arrest of growth in children.

3. Duration of the effect: β -adrenergic agonists, aminoxantines and antihistamines are mostly short-acting drugs, and as such must be administered frequently. Steroids which are long-acting drugs, have also long induction time and are of little value in emergencies.

The only existing mast cell specific drug is Cromolyn sodium. This drug can be used prophylactically, essentially without side effects. However, it has a very short half life, very long induction time, it can be applied only locally and only part of the patients respond to it. All these make use of Cromolyn sodium very limited.

A number of attempts to interfere with interaction between IgE and its high-affinity receptor, as a basis for anti-allergy therapy, have been reported in recent years. Recombinant peptides comprising structural elements from IgE (Helm, B., Kebo, D., Vercelli, D., Glovsky, M. M., Gould, H., Ishizaka, K., Geha, R., and Ishizaka, T. 1989. Blocking the passive sensitization of human mast cells and basophil granulocytes with IgE antibodies by a recombinant human ϵ -chain fragment of 76 amino acids. Proc. Natl. Acad. Sci. USA 86, 9465.) or FC ϵ RI (Ra, C., Kuromitsu, S., Hirose, T., Yasuda, S., Furuichi, K., and Okumura, K. 1993. Soluble human high affinity receptor for IgE abrogates the IgE-

mediated allergic reaction. *Int. Immunol.* 5, 47.; Haak-Frendscho, M., Ridgway, J., Shields, R., Robbins, K., Gorman, C., and Jardieu, P. 1993. Human IgE receptor α -chain IgG chimera blocks passive cutaneous anaphylaxis reaction in vivo. *J. Immunol.* 151, 351.) have been investigated as competitive inhibitors of the IgE-Fc ϵ RI interaction. Monoclonal antibodies generated against IgE (Baniyash, M., and Eshhar, Z. 1984. Inhibition of IgE binding to mast cells and basophils by monoclonal antibodies to murine IgE. *Eur. J. Immunol.* 14, 799) or Fc ϵ RI (Kitani, S., Kraft, D., Fischler, C., Mergenhagen, S. E., and Siraganian, R. P. 1988. Inhibition of allergic reactions with monoclonal antibody to the high affinity IgE receptor. *J. Immunol.* 140, 2585.), capable of blocking IgE binding to the receptor, without causing mast cell degranulation have also been tested. However, the affinity of IgE for Fc ϵ RI is very high ($K_M=10^{-10}M$), so that once it is bound to its receptor, the IgE molecule remains attached to the cell membrane for several weeks. Moreover, mast cell can be activated at low receptor occupancy: the cross-linkage of as few as 5% of receptors is sufficient to cause mast cell degranulation. These two properties of the system impede inhibition by competitive agents, thus limiting their clinical value. Our anti-allergy molecule depends to a much lesser extent on the ability to compete with IgE. Once having entered the target cell through a non-occupied IgE receptor, the chimeric protein affects the target cell. Moreover, early expression of the receptor in the maturation course of mast cells should allow the elimination of immature target cells before they are capable of mediator release. As the receptor is not expressed on stem cells, no damage to bone marrow is expected on the whole.

The IgE system is quite complex and diverse. Interactions between IgE and its binding structures have many functions apart from the allergic response, some of which are only beginning to emerge. Monoclonal antibodies against IL-4, the IL-4 receptor or the low-affinity IgE receptor eliminate expression of IgE in mice but have more general

immunosuppressive effects. The advantage of the present invention in which the high-affinity IgE receptor is targeted and not the overall IgE system, is therefore evident.

SUMMARY OF THE INVENTION

The present invention generally relates to a new approach for therapy of allergic responses, based on targeted elimination of cells expressing the FcεRI receptor by a chimeric cytotoxin FcεRI-PE₄₀. A sequence encoding amino acids 301-437 of the Fc region of the mouse IgE molecule was genetically fused to PE₄₀ - a truncated form of PE lacking the cell binding domain. The chimeric protein, produced in E. coli, specifically and efficiently kills mouse mast cell lines expressing the FcεRI receptor, as well as primary mast cells derived from bone marrow.

The present invention provides a chimeric protein for targeted elimination of FcεRI expressing cells especially useful for the therapy of allergic responses. The said chimeric protein is comprised of a cell targeting moiety for the FcεRI expressing cells and a cell killing moiety. The preferred killing moiety is the bacterial toxin Pseudomonas exotoxin (PE). This Pseudomonas exotoxin is a product of Pseudomonas aeruginosa.

The present invention also relates to a method for the preparation of said protein. This chimeric protein is prepared by genetically fusing the Fc region of the mouse IgE molecule to PE₄₀, a truncated form of PE lacking the cell binding domain.

The present invention also provides a pharmaceutical compositions, for the treatment of allergic diseases and for the treatment of hyperplasias and malignancies, comprising as an active ingredient the above mentioned chimeric protein and a conventional adjuvant product.

The present invention further relates to the method for the preparation of these pharmaceutical compositions comprising genetically fused Fc region of the mouse IgE molecule to PE₄ and adding, if needed, a conventional adjuvant product. The pharmaceutical compositions according to the present invention may be in any suitable form for injection, for topical application, or for oral administration.

DETAILED DESCRIPTION OF THE INVENTION

The Fc-PE chimeric protein according to the present invention has a number of advantages over the existing known drugs.:

1. Specificity: Fc-PE is highly specific, affecting the cells (mast cells and basophils) responsible for the release of allergic mediators. As it prevents the allergic attack, it can be of great value as a prophylactic treatment.

2. Toxicity: As it acts on effector cells and not on its target organs, Fc-PE is expected to have little, if any, side effects. Moreover, as the receptor is not expressed on stem cells, no damage to bone marrow and immunosuppression are anticipated. Re-institution of a normal physiological state is expected to occur within several weeks after the end of the treatment.

3. Duration of the effect: Because maturation of mast cells takes several weeks, the effect of Fc-PE is predicted to be long-standing, eliminating the need for frequent administration. Moreover, as in vitro studies indicate that reduction of 80% in cellular protein synthesis is observed in less than 4 hours, induction time of Fc-PE is expected to be relatively short, enabling its usage in acute phase allergic reactions.

Fcε-PE can also be valuable in the treatment of hyperplasias and malignancies of mast cells and basophils, like systemic mastocytosis (in both benign and malignant forms) and basophilic leukemia. Chemotherapy is not appropriate for patients with benign mastocytosis due to severe side effects. On the other hand, there is no good clinical protocol for the treatment of the malignant diseases. Fcε-PE chimeric protein, being highly potent and selective can be used for both benign and malignant conditions involving cells expressing the FcεRI receptors.

The following experimental results indicate that the $FC_{2-3-PE40}$ chimeric protein according to the present invention is a promising candidate for effective and selective allergy therapy.

The present invention provides a $Fc\epsilon$ -PE chimeric cytotoxin protein for the targeted elimination of $Fc\epsilon$ RI expressing cells, useful especially for the therapy of allergic responses such as asthma, allergic rhinitis, food allergies, atopic dermatitis, and anaphylaxis.

The said invention will be further described in detail by the following experiments. These experiments do not intend to limit the scope of the invention but to demonstrate and clarify it only.

1. Construction of $Fc\epsilon$ -PE₄₀ chimeric proteins.

For the targeting moiety of the chimeric proteins fragments of the mouse IgE constant region ($Fc\epsilon$) are used as it binds both to human and to mouse high affinity IgE receptors (Conrad, D. H., Wingard, J.R., and Ishizaka, T. 1983 The interaction of human and rodent IgE with the human basophil IgE receptor. J. Immunol. 130, 327.).

We used a sequence corresponding to a.a. 301-437, containing the COOH terminus of domain 2 and the entire domain 3 (C_2 '- C_3). We used also a sequence corresponding to a.a. 225-552, containing the whole C_2 - C_4 domains. The cDNA for these fragments was obtained by RT-PCR, using RNA isolated from mouse B cells which were isotopically switched to secrete IgE and a specific set of primers. B cells obtained from the spleen of a 6-week-old BALB/C mouse were separated by negative selection using anti-Thy1.2 and rabbit complement. Cells were incubated at 2×10^6 cells/ml in the presence of Lipopolysaccharide (LPS, $10 \mu\text{g/ml}$) and IL_4 (500 u/ml) for 5 days to induce isotypic switching for IgE production. After 5 days, total cellular RNA was isolated (RNAzol TM B isolation kit produced by BIOTECK Laboratories,

Houston, USA.). Total RNA (2.5 µg) was then reverse transcribed into first strand cDNA, using the reverse transcription System (Promega, USA) under conditions, recommended by the manufacturer. The cDNA was diluted to a total volume of 1 ml with TE buffer (10 mM Tris-HCL, pH 7.6, 1 mM EDTA) and stored at 4°C until used.

Fcε fragments were generated by PCR, using cDNA and a pair of synthetic oligonucleotide primers 5'-GCG GAT CCC ATA TGG AGC AAT GGA TGT CGT-3' (sense, starting from nucleotide 406, according to gene bank sequence J00476) and 5'-GCG GAT CCC ATA TGT GGG GTC TTG GTG ATG GAA C-3' (antisense, starting from nucleotide 813) for the Fcε₂₋₃ sequence and 5'-GCG GAT CCC ATA TGC GAC CTG TCA ACA TCA CTG-3' (sense, starting from nucleotide 175) and 5'-GCG GAT CCC ATA TGG GAG GGA CGG AGG GAG G-3' (antisense, starting from nucleotide 1167) for the Fcε₂₋₄ sequence.

Synthetic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified on oligonucleotide purification cartridges. The vent polymerase enzyme (Biolabs) was used for amplification. The reaction mixture was incubated in a DNA thermal cycler (MJ Research, Inc, USA.) for 33 cycles. Each cycle consisted of 1 min. at 95°C, 1 min. at the annealing temperature and 2 min. at 72°C. The MgSO₄ concentration and the annealing temperature used for each primer pair were: 2.5 mM and 61°C for Fcε₂₋₃, 2 mM and 57°C for Fcε₂₋₄.

The pHL 906 plasmid, which encodes IL₂-PE₄₀, was described previously (Fishman, A., Bar-Kana, Y., Steinberger, I., and Lorberboum-Galski, H. 1994. Increased cytotoxicity of IL2-PE chimeric proteins containing targeting signal for lysosomal membranes. Biochem. 33, 6235.). The pHL906 plasmid was cut with NdeI, obtaining the larger fragment of 3596 bp. The above Fcε fragment was inserted into the NdeI site of pHL906. The resulting plasmids, pAF2302 and pAF2415, coding for the C₂'-C₃ and C₂-C₄ fragments respectively, each fused 5' to PE₄₀, were characterized by restriction and sequence analysis

(results not shown). *Escherichia coli* strain HB101 was used for transformation and preparation of the plasmids.

2. Expression and partial purification of the chimeric proteins.

The newly designed chimeric protein, Fcε-PE₄₀ encoded by plasmid pAF2302 was expressed in *E. coli* strain BL21(lambda-DE3) which carries a T7 RNA polymerase gene in a lysogenic and inducible form. Induction was performed at O.D.₆₀₀ 0.5 for 180 min. in the presence of isopropyl β-D-thiogalactoside (IPTG, 1mM final concentration). A pellet expressing cells was suspended in TE buffer (50 mM Tris pH 8.0, 1mM EDTA) containing 0.2 mg/ml lysosyme, sonicated (three 30s bursts) and centrifuged at 30,000Xg for 30 min. The supernatant (soluble fraction) was removed and kept for analysis. The pellet was denatured in extraction buffer (6 M guanidine-hydrochloride, 0.1 M Tris pH 8.6, 1mM EDTA, 0.05 M NaCl and 10 mM DTT) and stirred for 30 min. at 4°C. The suspension was cleared by centrifugation at 30,000Xg for 15 min. and the pellet discarded. The supernatant was then dialysed against 0.1 M Tris (pH 8.0), 1 mM EDTA, 0.25 mM NaCl and 0.25 mM L-Arginine for 16 h. The dialysate was centrifuged at 15,000Xg for 15 min. and the resultant supernatant (insoluble fraction, guanidine-hydrochloride treated) was used as a source of the chimeric proteins. Proteins were characterized by gel electrophoresis (Fig. 2). The protein profile of whole cell extracts revealed the high expression level of the chimeric protein.

The protein was further characterized by Western blot analysis using antibodies against PE (Fig. 3A) and against IgE (Serotec, England) (Fig. 3B). The electrophoresed samples were transferred onto nitrocellulose and immunoblotted as described (Lorberboum-Galski, H., Fitzgerald, D.J., Chaudhary, V., Ashya, S., and Pastan, I. 1988. Cytotoxic activity of an interleukin 2 - *Pseudomonas* exotoxin chimeric protein produced in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 85, 1992.). A Vectastain ABC Kit

(Vector Laboratories, USA) was used according to the manufacturer's instructions. The chimera reacted with both antibodies, thus confirming the cloning and production of in-frame full-length chimeric protein.

Subcellular fractionation of expressing cells revealed that the insoluble fraction (inclusion bodies) was particularly rich with chimeric protein (Fig. 2). This fraction was therefore used as the source of the chimeric protein.

The ADP-ribosylation activity of tested samples was measured using wheat germ extracts enriched in elongation factor 2 as substrate, as described previously, and revealed that the novel chimeric protein was enzymatically active (results not shown).

3. Effect of FC_{2-3} -PE₄₀ chimeric protein on mouse mast cell lines.

The cytotoxic effect of the chimeric protein was tested on various mouse mast cell lines known to express the $FC_{\epsilon}RI$ receptor. The cytotoxic activity of the chimeric protein was evaluated by inhibition of protein synthesis, as measured by [³H] Leucine incorporation. Various concentrations of the chimeric protein, diluted with 0.25% bovine serum albumin in phosphate-buffered saline, were added to 2×10^4 cells/0.2 ml seeded in 96-well plates for 20h., followed by an 8h pulse with 2 μ Ci of [³H]-Leucine. The results are expressed as a percentage of the control experiments in which the cells were not exposed to the chimeric protein. All assays were carried out in triplicate in three separate experiments.

Three target cell lines expressing the $FC_{\epsilon}RI$ receptor were used: MC-9, a mast cell line originating in mouse fetal liver and dependent on IL₃ for growth, C57, an IL₃ independent mast cell line originating in mouse bone marrow; and the Abelson-virus transformed mast cell line originating in mouse midgestation embryonic placenta.

Fcε-PE₄₀ was found to be cytotoxic in a dose-dependent manner to all the cell lines tested (Fig. 4). The MC-9 and C57 lines were extremely sensitive to the chimeric toxin, with an ID₅₀ of 50-75 ng/ml and 100-125 ng/ml, respectively. The Alelson cell line was much less sensitive (ID₅₀ of 1200-1500 ng/ml).

4. Specificity of Fcε-PE₄₀ response.

To verify the specificity of FC₂₋₃-PE₄₀ activity, two control proteins, PE₄₀ and FC₂₋₃-PE_{40M}, were generated and evaluated for their effect on target and non target cells. To construct FC₂₋₃-PE_{40M}, the region coding for the 122 amino acids at the C-terminal of PE was excised with EcoRI and BamHI and replaced by a corresponding fragment carrying a deletion at amino acid 553.

PE₄₀, which has no intrinsic targeting capacity had, as expected, no effect on the target cell lines (Fig. 4). FC₂₋₃-PE_{40M} which possesses a FC₂₋₃ moiety linked to a mutated, enzymatically inactive form PE₄₀, was also not cytotoxic to the target cells (Fig. 4).

In addition, it was possible to block the cytotoxic effect of FC₂₋₃-PE₄₀ against target cells by whole mouse IgE (40 µg/ml, Fig. 5A) or by a αPE polyclonal antibody (10 µg/ml, Fig. 5B).

The effect of FC₂₋₃-PE₄₀ was also tested on various mouse non-target cell lines (Table 1). All cell lines of hemopoietic origin were unaffected by the chimeric protein. Surprisingly, fibroblast and hematoma cell lines exhibited some sensitivity to chimeric toxin, although the ID₅₀ values were twenty-fold higher than those of the MC-9 cells (Table 1).

The above data demonstrates that the toxic effect of FC₂₋₃-PE₄₀ on mast cell lines is due to a specific response mediated by the FC₂₋₃ moiety which targets the cytotoxic

part of the chimera (PE₄₀) into the cell.

5. Effect of chimeric proteins on primary mast cells.

As it is likely that fresh murine mast cells react differently from established cell lines, we also tested primary mast cells obtained from normal mice for their sensitivity to FC₂₋₃-PE₄₀. When cultured in the presence of IL₃ for two weeks, mouse bone marrow differentiates into an almost pure population of cells with the morphology of immature mast cells, containing granules and expressing the FcεRI receptor.

BALB/C mice aged 4-6 weeks were sacrificed and their bone marrow was aseptically flushed from femurs into 0.9% cold NaCl. The cell suspension was washed twice with 0.9% NaCl, centrifuged for 10 min. at 300Xg and finally resuspended in RPMI 1640 medium containing 10% heat inactivated fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5x10⁻⁵ M β-mercaptoethanol, 100 u/ml penicillin, 100 µg/ml streptomycin and 20 u/ml recombinant mouse IL₃. Cells were grown in tissue culture flasks at a density of 10⁶ cells/ml, at 37°C in a 5% CO₂ humidified atmosphere for 2-3 weeks. The media were changed every 7 days. Recombinant IL₄ (10u/ml) was added starting from day 7 in culture.

To follow the degree of maturation, cells were mounted on slides, stained with acidic Toluidine Blue (pH 1.0) and examined microscopically under oil.

The effect of chimeric proteins was tested on bone marrow derived mast cells (BMMC) on the 16th day of culture. As shown in Fig. 6, FC₂₋₃-PE₄₀ was cytotoxic to BMMC in a dose dependent manner, with an ID₅₀ of 125 ng/ml. At a high chimeric protein dose, there was nearly 100% inhibition of protein synthesis. None of the control proteins FC₂₋₃-PE_{40M} or PE₄₀ displayed cytotoxicity against BMMC (Fig. 6). Thus, primary mast cells respond towards the chimeric

protein similarly to the established mast cell lines (Fig. 4 and 6).

6. Receptor specificity of FC_{2-3} -PE₄₀.

Aside from the high affinity $Fc\epsilon RI$ receptor, three other membrane surface structures were reported to bind IgE with low affinity - the low affinity $Fc\epsilon RII$ receptor, the ϵBP galactoside-binding protein (also termed MAC-2 or CBP35) and the $Fc\gamma RII/III$ receptor. These structures appear on various cell types, mainly of hemopoietic origin, but also on fibroblasts (ϵBP). $Fc\gamma RII/III$ and ϵBP

appear on mast cell membranes in addition to $Fc\epsilon RI$. As our aim was to target only mast cells, it was essential to prove that the chimeric protein does not recognize these structures and thus can not be internalized through them. Theoretically our chimeric protein does not fulfill the binding requirements of the low-affinity IgE binding structure $Fc\epsilon RII$, ϵBP and $Fc\gamma RII/III$. $Fc\epsilon RII$ binds only disulfide linked ϵ -chain dimers, while our protein lacks domain 4 which is essential for dimerization. ϵBP binds only glycosylated IgE; FC_{2-3} -PE₄₀ being produced in bacteria, is not glycosylated. $Fc\gamma RII/III$ binds IgE-immunocomplexes but not free IgE. Nevertheless, the issue of receptor binding was challenged experimentally.

Experiments involving ϵBP and $Fc\gamma RII/III$ were performed on C57 mast cells, known to express these receptors in addition to $Fc\epsilon RI$. To test whether the chimeric protein can enter the cell via the $Fc\gamma RII/III$ receptors, cells were preincubated with the 2.4G2 antibody (Pharmigen) (50 $\mu g/m$) prior to addition of the chimeric protein. This monoclonal antibody, which binds to the extracellular domains of both $Fc\gamma RII$ and the $Fc\gamma RIII$ receptors was shown to be a competitive inhibitor of IgE binding. As can be seen in Fig. 7A, there was no difference in the cellular response to FC_{2-3} -PE₄₀ between control cells and cells preincubated with the antibody.

We next examined whether ϵ BP is involved in the cytotoxicity of $FC_{2-3}-PE_{40}$. As ϵ BP is attached to membrane carbohydrate determinants, addition of lactose to the culture medium causes its dissociation from the cell surface. We found no difference in the cellular response to $FC_{2-3}-PE_{40}$ in the presence or absence of lactose (25mM, Fig. 7B).

Additional experiments in the presence of 2.4G2 antibody and lactose were performed on fibroblast cell lines that were found partially responsive to the chimeric protein (Table 1). Again, there was no difference in $FC_{2-3}-PE_{40}$ cytotoxicity against treated and control cells (results not shown).

To test whether $FC_{2-3}-PE_{40}$ affects $FC_{\epsilon}RII$ -bearing cells, we used the 0.12A3 cell line, a mouse B cell hybridoma expressing the $FC_{\epsilon}RII$ receptor. The 0.12A3 cells were totally non responsive to $FC_{2-3}-PE_{40}$, even at high doses (>5000 ng/ml, Fig. 8A). As this line loses the receptor upon long term culture, the assay was followed by FACS analysis with the B3B4 antibody against the receptor (Pharmingen). The results showed that the receptor was expressed on 54% of the cells (results not shown).

An additional experiment was performed on fresh mouse B splenocytes preincubated for 16 h. with LPS (50 μ g/ml) to stimulate expression of $FC_{\epsilon}RII$. $FC_{2-3}-PE_{40}$ has no effect on these B splenocytes (Fig. 8B), although 69% of the cells expressed the receptor, as determined by FACS analysis.

Collectively, these results suggest that $FC_{2-3}-PE_{40}$ does not bind to the low affinity IgE-binding structures, namely $FC_{\epsilon}RII$, $FC_{\delta}RII/III$ and ϵ BP.

7. Effect of FC_{2-3} -PE₄₀ on cellular degranulation.

Because of the possible clinical applicability of FC_{2-3} -PE₄₀, it was important to test whether treatment of mast cells with FC_{2-3} -PE₄₀ results in the release of allergic mediators triggered upon $FC_{\epsilon}RI$ binding by the chimeric protein.

C57 cells prelabelled overnight with [³H]-hydroxytryptamine (10 μ ci/ml) were washed, plated at 2×10^5 cells/well in DMEM containing 10% FCS, in 96-well tissue culture plates and incubated with FC_{2-3} -PE₄₀ (10 μ g/ml) at 37 °C. At various time points, supernatants were separated and release of serotonin into the supernatant was measured. Unlabelled cells were also incubated with FC_{2-3} -PE₄₀ and at the same time intervals were pulsed 1 hr with [³H] leucine to measure protein synthesis inhibition by chimeric toxin. There was no difference in supernatant [³H] serotonin content between FC_{2-3} -PE₄₀ treated and untreated cells at $\frac{1}{2}$, 4 or 8 hr following chimeric protein addition (Fig. 9A). Inhibition of protein synthesis reached 80% at 4 h. and a value of 90% by 8 h. (Fig. 9B). These results suggest that FC_{2-3} -PE₄₀ does not cause release of allergic mediators during receptor binding or upon inhibition of protein synthesis.

8. Electrophoretic characterization of Fc ϵ -PE40

Western blot analysis of electrophoresed samples run under non-reducing conditions (omitting 2-mercaptoethanol from the sample buffer) revealed that the Fc2'-3-PE40 chimeric protein is predominantly present as a monomer (figure 10b). For native PAGE, 2-mercaptoethanol was omitted from the sample buffer and the samples were not heated. In addition, SDS was replaced with equivalent volumes of water in the gel, sample buffer and electrode running buffer. Under non-denaturing conditions the chimeric protein runs as a broad band (figure 10c). A single native system can not distinguish the effects of molecular weight, charge and conformation on protein electrophoretic mobilities. However, the proximity of the molecules in the band indicates that they can not differ much in these parameters.

9. Internalization assay

In vitro activity of the chimeric protein is achieved only upon its internalization. To test whether the chimeric protein is internalized, 5×10^5 cells/3ml were incubated for 1 hour with 20 μ g of the chimeric protein at 37°C. After 3 washes with cold PBS the pellet was treated with 0.5 ml of acid solution (0.15M NaCl, 0.15M acetic acid (pH 3)) for 3 min on ice to remove membrane-bounded chimeric protein. The pH was then neutralized by addition of 50% FCS following by three washes with RPMI/10% FCS. The cell pellet was lysed with 0.3 ml of RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 15% SDS, 1% deoxycholic acid, 1% Nonidet P-40). Various samples were electrophoresed and immunoblotted using α -PE and the ECL detection system (Amersham). Western blot analysis revealed undoubtedly that Fc2'-3-PE40 chimeric protein is internalized into the target cells (figure 11).

10. Effect of FC_{2-3} -PE₄₀ on cellular degranulation

C57 cells were incubated overnight with [³H]-Hydroxytryptamine (10μci/ml) at 37°C. Cells were washed 3 times to remove free [³H]-Hydroxytryptamine, plated in Tyrod's buffer (10mM Hepes pH 7.4, 130 mM NaCl, 5 mM KCl, 5.6 mM Glucose, 0.5% BSA) at 2.5×10^5 cells/0.5 ml in 24 well tissue culture plates and incubated with IgE (10μg/ml) for 1 hour at 4°C. MgCl₂ and CaCl₂ were then added to the final concentration of 1 mM and 1.6 mM respectively, following by incubation with Dinitrophenyl-human serum albumin (DNP-HSA, 50 ng/ml) for 30 minutes or with the different concentrations of chimeric protein for various times at 37°C. Cell-free supernatants were collected by centrifugation and amount of [³H]-Hydroxytryptamine released was measured. No degranulation was observed with any concentration of chimeric protein tested (figure 12a). As a control, cells preincubated with IgE were exposed to DNP under the same conditions. The effect of triggering degranulation by DNP is clearly visible (figure 12a). FC_{2-3} -PE₄₀ did not cause any degranulation also at later stages of it's interaction with the target cell (figure 12b), while it inhibits protein synthesis by over 80% (figure 12c). Our results demonstrate that FC_{2-3} -PE₄₀ does not trigger degranulation at any stage during it's interaction with the cell.

CLAIMS

1. A chimeric protein for the therapy of allergic responses by the way of targeted elimination of Fc ϵ RI expressing cells wherein the said chimeric protein is comprised of a cell targeting moiety for the Fc ϵ RI expressing cells and a cell killing moiety.
2. A chimeric protein according to claim 1 wherein the killing moiety is the bacterial toxin Pseudomonas exotoxin (PE).
3. A chimeric protein according to claim 1 wherein the cell targeting moiety is the Fc region of the mouse IgE molecule.
4. A chimeric protein according to claim 1 wherein the cell targeting and cell killing moieties are genetically fused.
5. A chimeric protein according to claim 1 wherein a sequence encoding amino acids 225-552 of the Fc region of the mouse IgE molecule is genetically fused to PE $_{\Delta 0}$, a truncated form of PE lacking the cell binding domain.
6. A chimeric protein according to claim 1 wherein a sequence encoding amino acids 301-437 of the Fc region of the mouse IgE molecule is genetically fused to PE $_{\Delta 0}$, a truncated form of PE lacking the cell binding domain.
7. A chimeric protein as defined in claim 1 for use in allergic diseases selected from asthma, allergic rhinitis, food allergies, atopic dermatitis, and anaphylaxis.

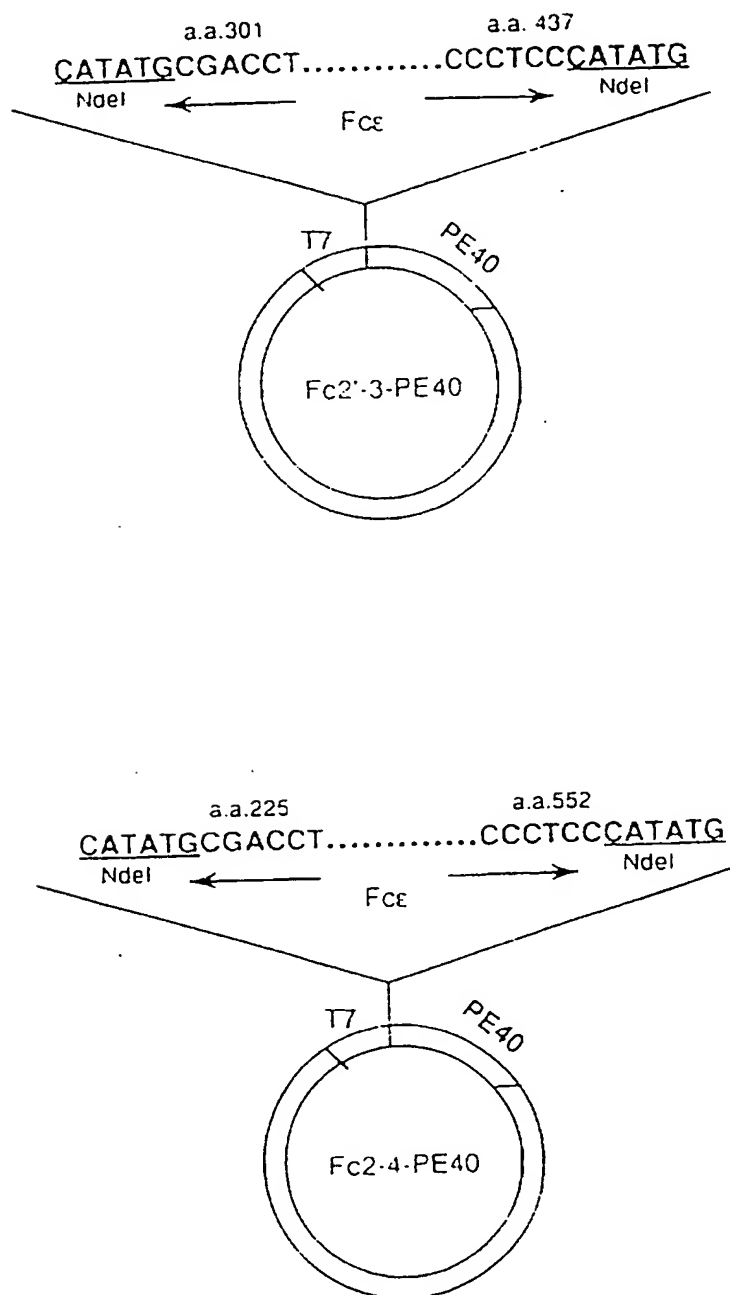
8. Pharmaceutical compositions for the treatment of allergic diseases and for the treatment of hyperplasias and malignancies evolving cells expressing Fc ϵ RI receptor comprising of an active ingredient a chimeric protein as defined in claims 1-6 and a conventional adjuvant product.

9. A pharmaceutical composition for the treatment of allergic diseases and for the treatment of hyperplasias and malignancies according to claim 8 wherein said composition is in a suitable form for injection (intra-veneous, intra-articular, sub-cutaneous, intra-muscular, intra-peritoneal), intra-nasal, intra-theccal, intra-dermal, trans-dermal, inhalation, topical application, oral administration, sustained release, or by any other route including the enteral route.

10. A method for the preparation of a pharmaceutical composition as defined in claim 9 comprising genetically fusing the Fc region of the mouse IgE molecule to PE and adding if needed a conventional adjuvant product.

11. A plasmid comprising a promoter operably linked to a DNA molecule encoding a peptide as defined in claims 1-5.

Figure 1: Schematic representation of plasmids coding for the Fc₂.₃-PE₄₀ and Fc₂.₄-PE₄₀ chimeric proteins.



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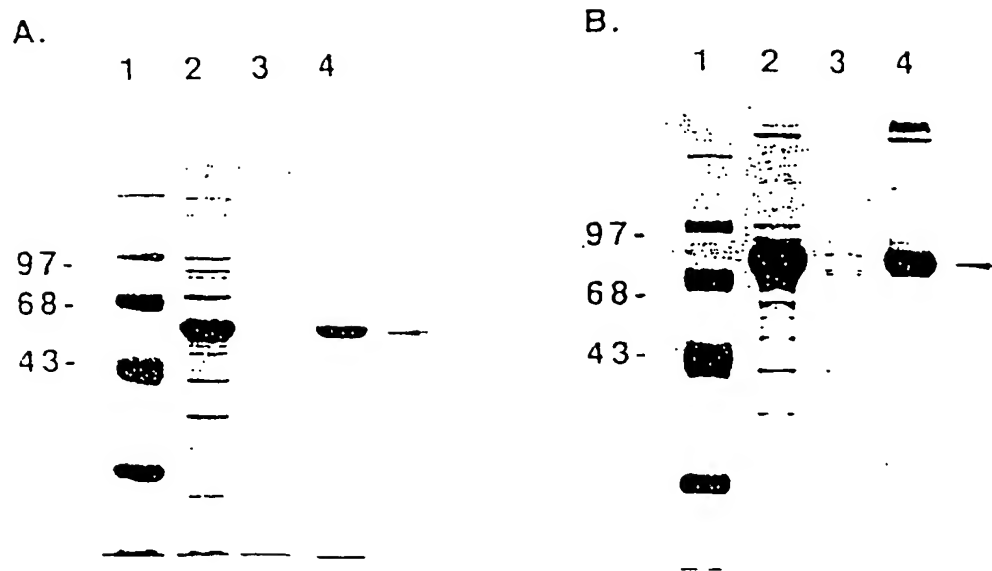


Figure 2: SDS-polyacrylamide gel electrophoresis analysis of cell fractions containing Fc ϵ -PE₄₀ chimeric proteins. Samples containing 5 μ g. total protein were loaded onto 10% gels. (A) Cells expressing Fc_{2.3}-PE₄₀. Lane 1, markers; Lane 2, whole cell extract; Lane 3, soluble fraction; Lane 4, insoluble fraction. (B) Cells expressing Fc_{2.4}-PE₄₀. Fractions are as described in A.

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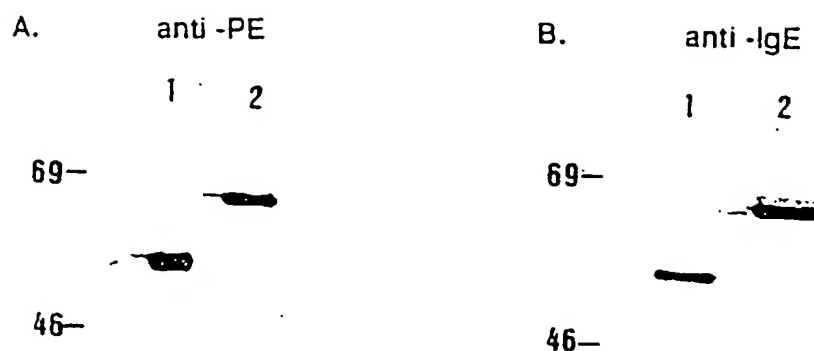


Figure 3: Immunoblotting of insoluble fractions containing Fc ϵ -PE₄₀ chimeric proteins with antibodies against PE (A) and IgE (B). Samples containing 1 μ g of total protein were loaded onto 10% SDS-polyacrylamide gels. Electrophoresed samples were transferred onto nitrocellulose and processed as described in Materials and Methods. (A) α PE: Lane 1, Fc₂₋₃-PE₄₀; Lane 2: Fc₂₋₄-PE₄₀. (B) α IgE: Lanes are as described in A.

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4A.

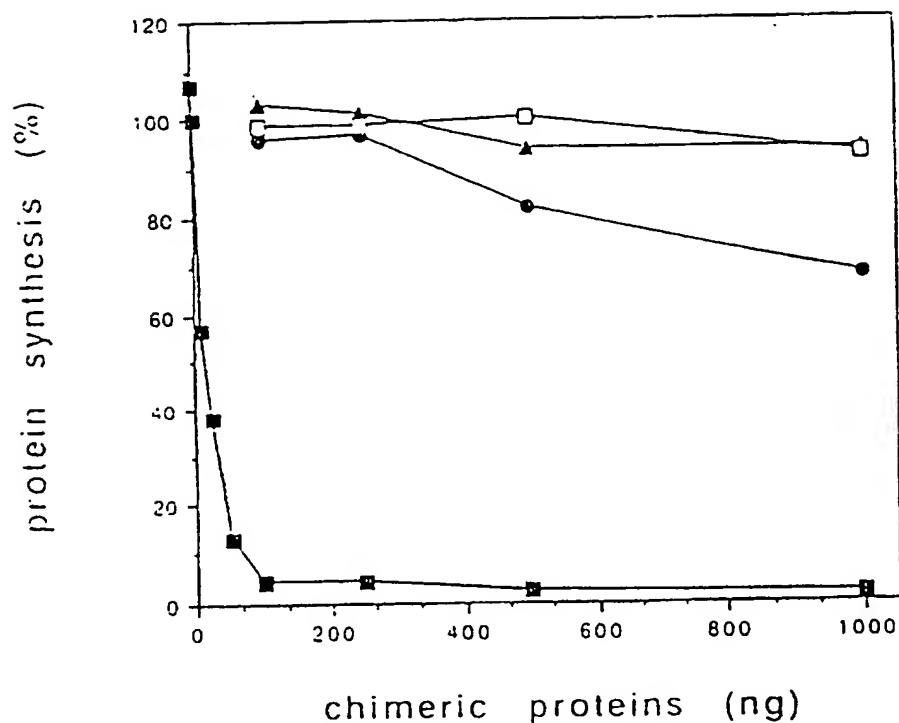
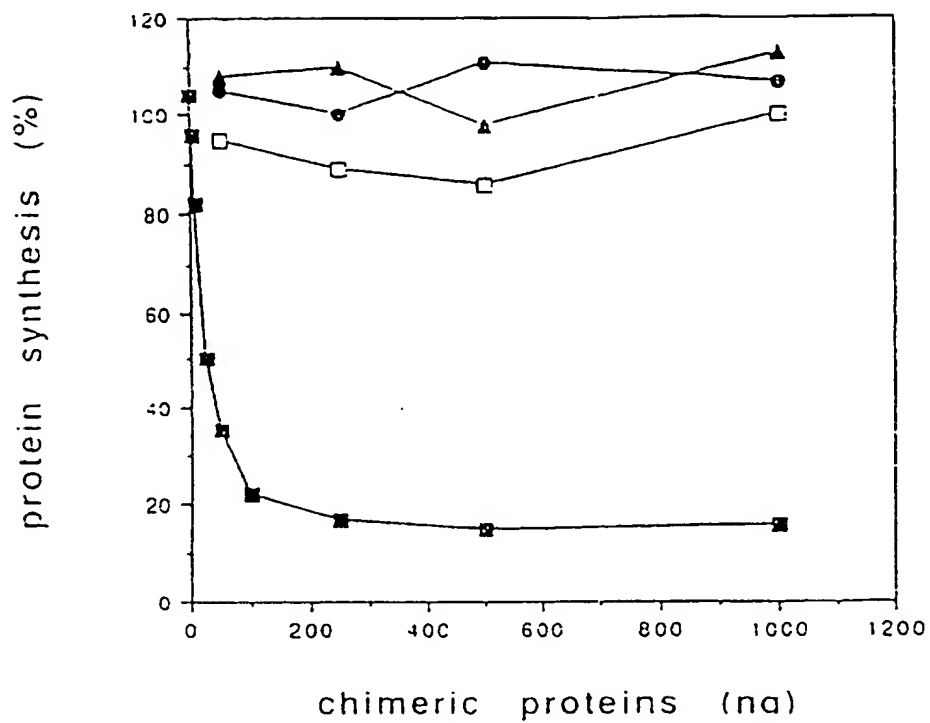


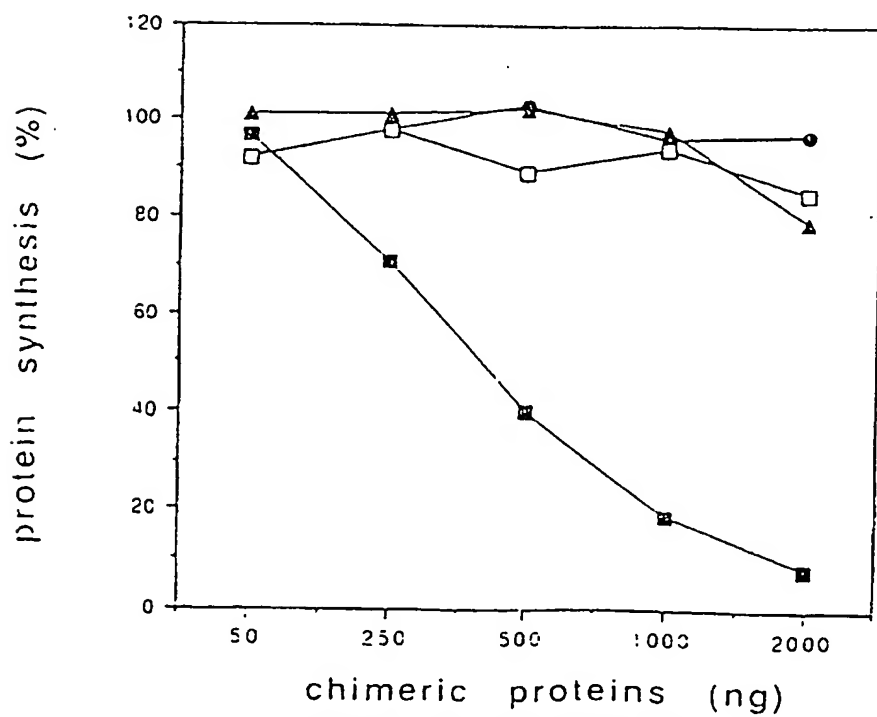
Figure 4: Cytotoxic activity of various chimeric proteins against MC-9 cells (A), C57 cells (B), and Abelson cells (C). Cells were incubated for 20 h with insoluble fractions containing the chimeric proteins -■- Fc₂₋₃-PE₄₀; -●- Fc₂₋₄-PE₄₀; -▲- Fc₂₋₃-PE_{40M} or -□- PE₄₀ at various concentrations (according to total protein concentration). Experiments with MC-9 cells were performed in the presence of IL₃ (20 u/ml) and IL₄ (10 u/ml). [³H] Leucine incorporation into cell proteins was measured as described in Materials and Methods. The results are expressed as the percentage of protein synthesis of control cells not exposed to chimeric proteins.

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4B.



4C.



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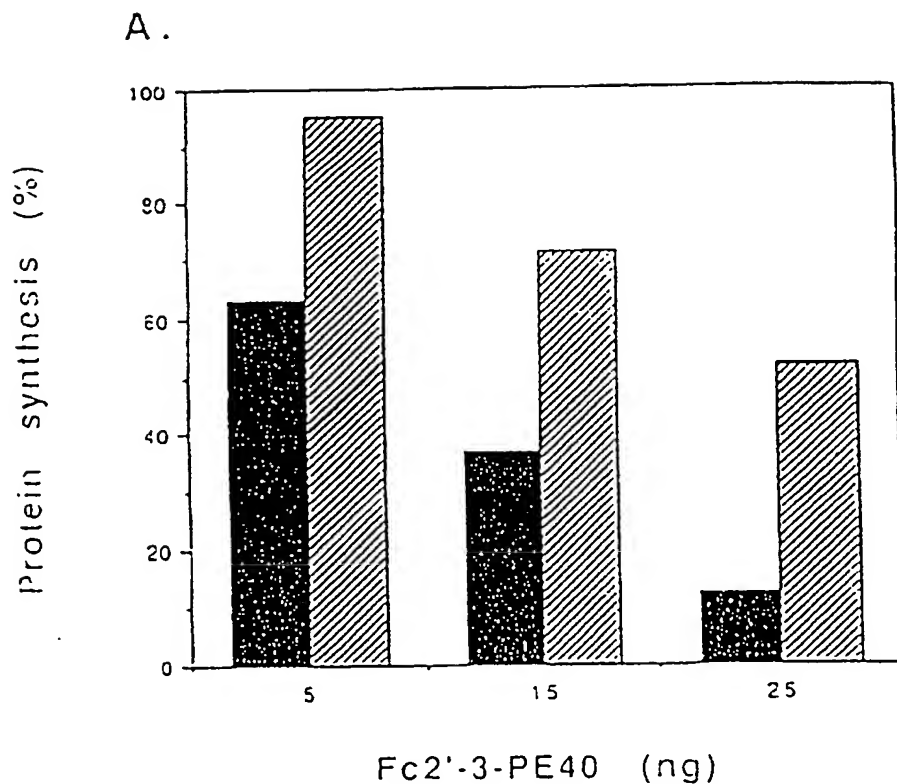
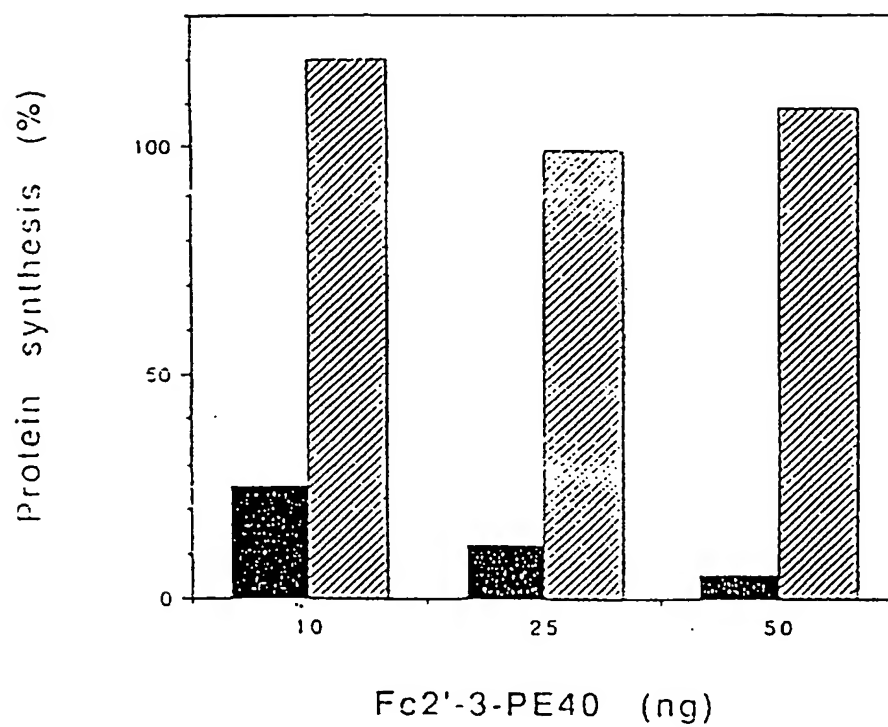


Figure 5: Inhibition of $\text{Fc}_{2'-3}\text{-PE}_{40}$ cytotoxicity by (A) IgE and (B) αPE . Cells were incubated with whole IgE (40 mg/ml) for 1 h at 4°C before the addition of $\text{Fc}_{2'-3}\text{-PE}_{40}$. αPE (10 mg/ml) was added a few minutes prior to the addition of $\text{Fc}_{2'-3}\text{-PE}_{40}$. All other experimental conditions were as described in Figure 4. ■

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5 B.



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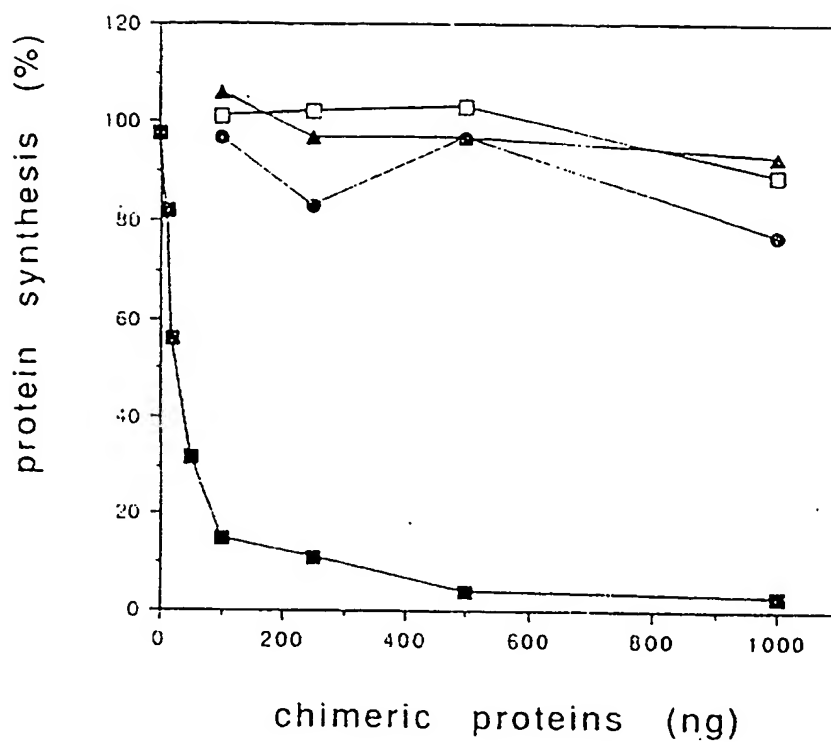


Figure 6: Cytotoxic activity of various chimeric proteins against bone marrow derived primary mast cells (BMMC). Bone marrow was cultured as described in Materials and Methods. Experiments were performed on the 16th day of culture, as described in Fig 4, in the presence of IL₃ (20 u/ml) and IL₄ (10 u/ml).

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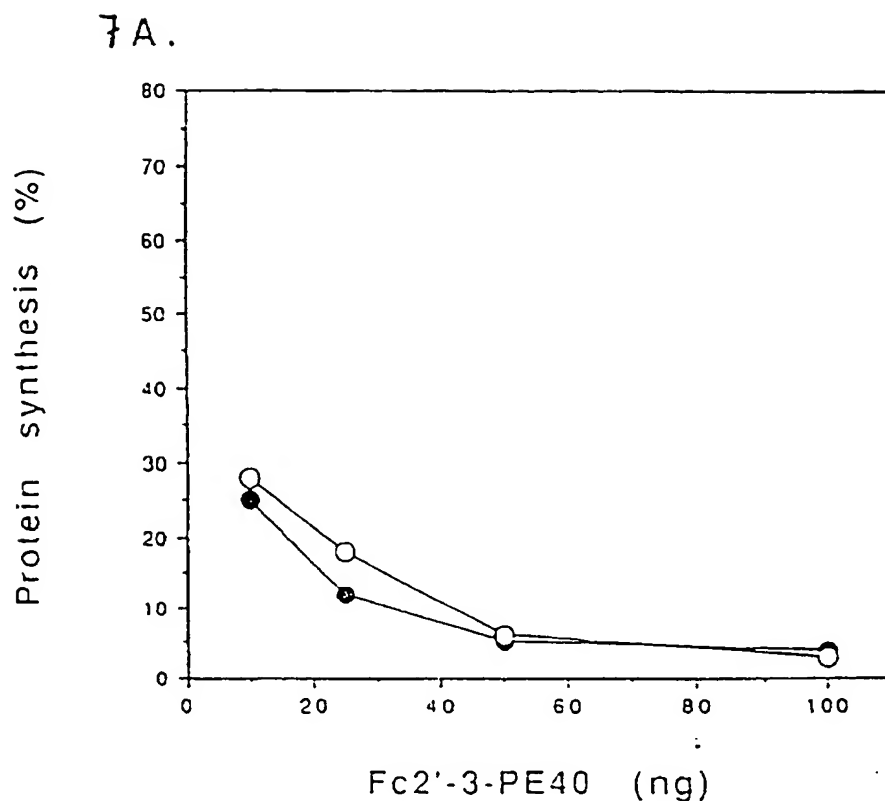
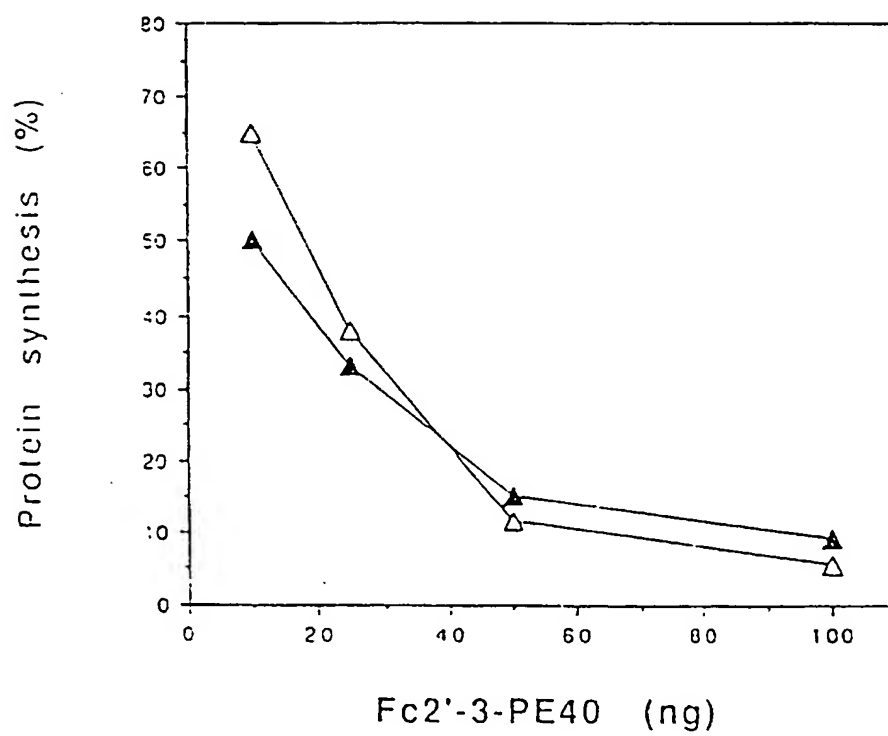


Figure 7A. Cytotoxic activity of various chimeric proteins against the C57 cells in the presence of $\alpha\text{Fc}\gamma\text{RII/III}$ (2.4G2). Cells were incubated with 2.4G2 (50 $\mu\text{g/ml}$) or galactose (25 mM) for 30 min. at 37°C prior to the addition of $\text{Fc}_{2'-3}\text{-PE}_{40}$. All other experimental conditions were as described in Figure 4. (A) $\text{Fc}_{2'-3}\text{-PE}_{40}$ in the absence (●) or presence (○) of 2.4G2. (B): $\text{Fc}_{2'-3}\text{-PE}_{40}$ in the absence (Δ) or presence (\blacktriangle) of galactose.

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7 B.



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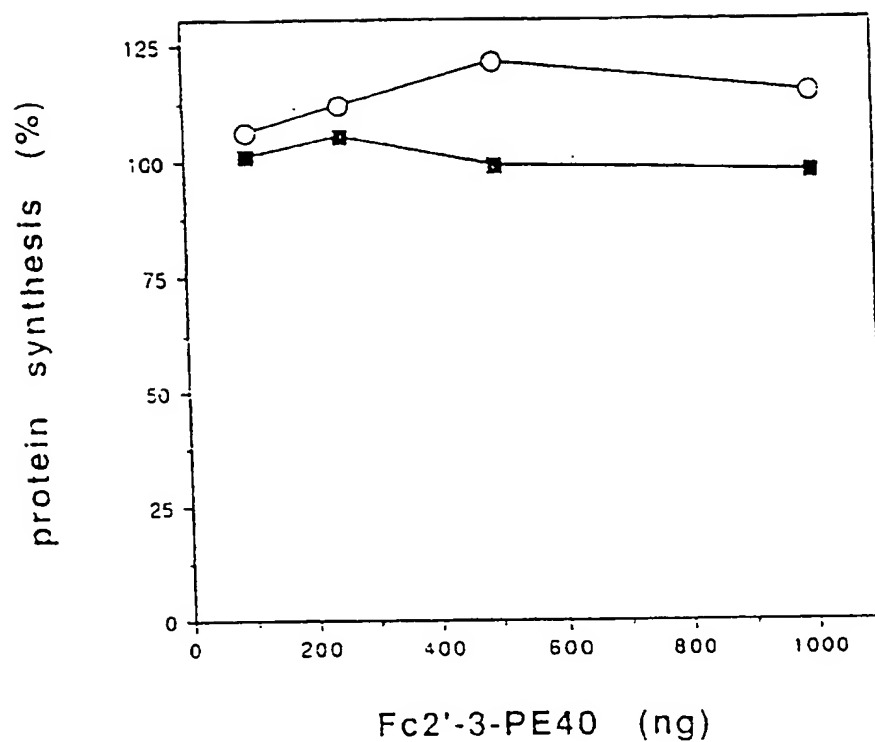


Figure 8: Cytotoxic activity of various chimeric proteins against FcεRII bearing cells. -○- B splenocytes. -■- 0.12A3 B cell hybridoma. B splenocytes were preincubated for 16h. with LPS (50μg/ml) and IL₄ (50u/ml). All other experimental conditions were as described in Figure 4.

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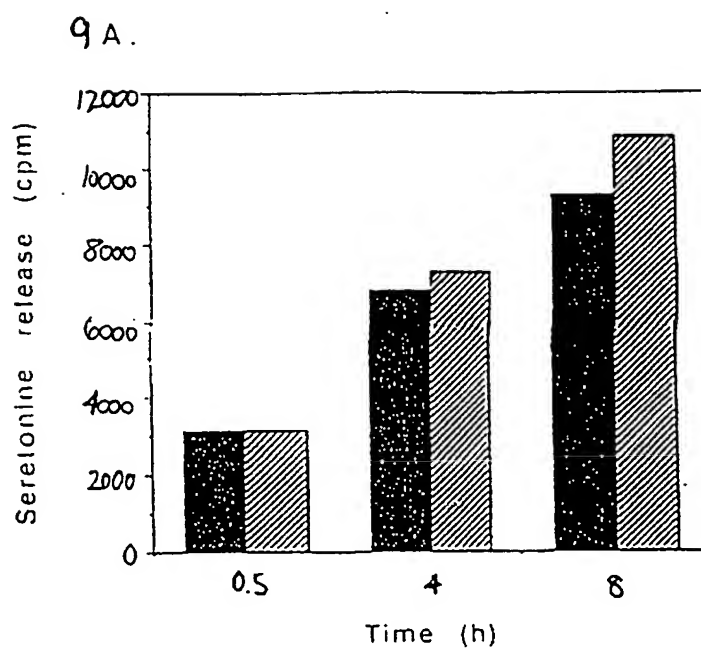


Figure 9: (A): The effect of $Fc_{2.3}$ -PE₄₀ on serotonin release from CS7 cells. Cells were labeled overnight with [³H] Hydroxytryptamine creatinine sulfate. The cells were then washed and incubated with $Fc_{2.3}$ -PE₄₀ (10μg/ml). Control cells were not exposed to any protein. At different time points [³H] Hydroxytryptamine creatinine sulfate release into the medium was measured. ■- control, ▨ $Fc_{2.3}$ -PE₄₀.

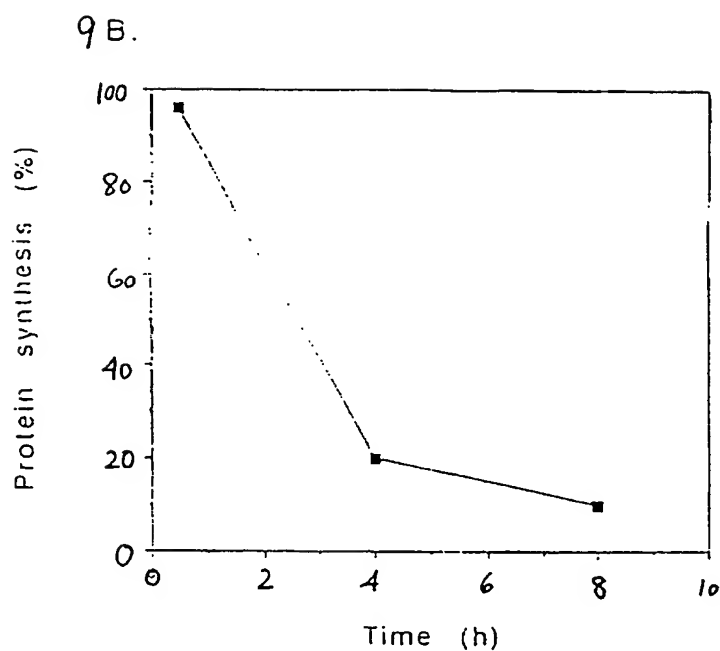
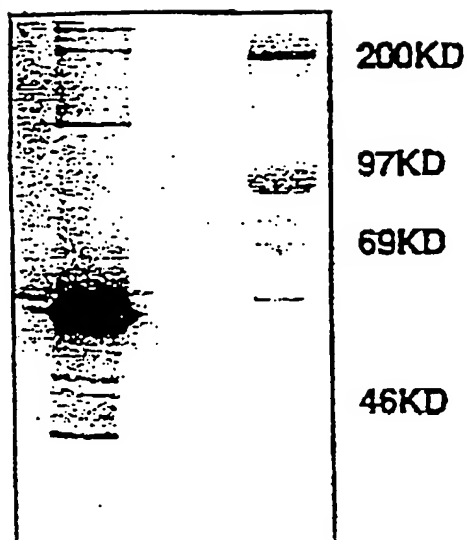


FIGURE 9 (B): Time-dependant cytotoxicity of $Fc_{2.3}\text{-PE}_{40}$ against C57 cells.

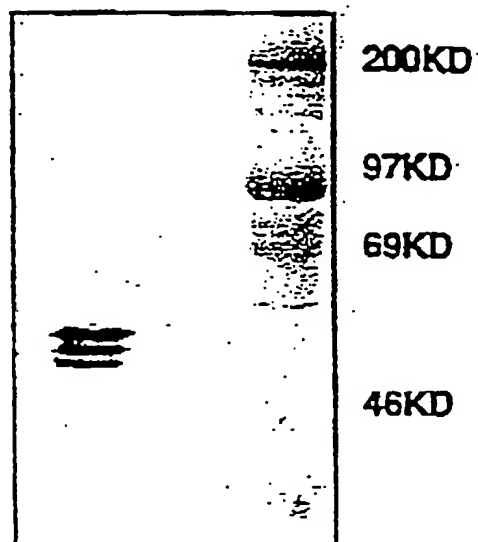
Unlabeled cells were incubated as in (A). At the same time points, cells were pulsed for 1 h with [^3H] Leucine and its incorporation into cellular proteins was measured. The results are expressed as the percentage of protein synthesis of control cells not exposed to chimeric proteins.

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10A.



10B.



10 C.

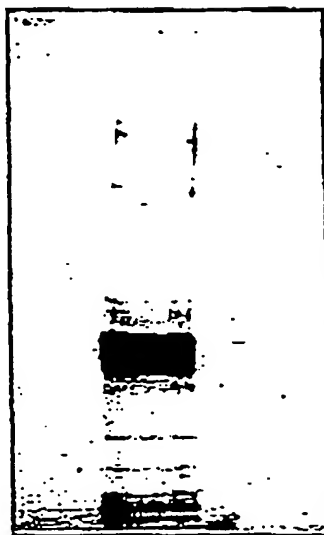


FIGURE 10 Immunoblotting of FC_{2-3} -PE40 chimeric protein electrophoresed under the following conditions with anti-PE: A) in SDS under reducing conditions, B) in SDS under nonreducing conditions and C) a nondenaturing gel (i.e. no reduction, no SDS).

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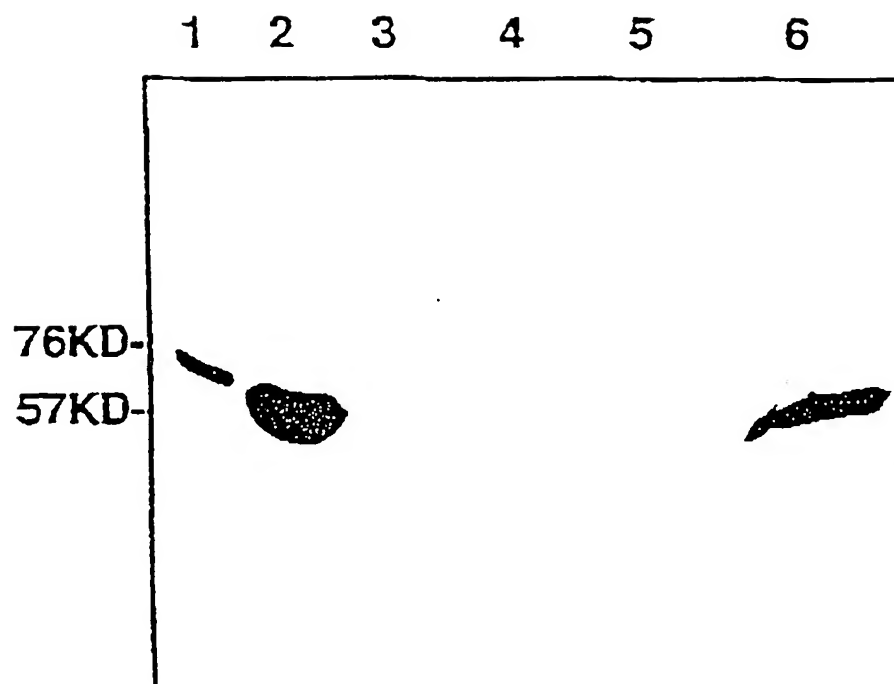


FIGURE 11 Internalization of Fc_{2-3} -PE40 chimeric protein by MC-9 cells. Samples containing 20 μl of each of the following fractions were loaded onto SDS-10% polyacrylamide gels: lane 1, 40 ng Fc_{2-3} -PE40; lane 2, supernatant of the cells; lane 3, last wash before the acid treatment; lane 4, acid wash supernatant; lane 5, last wash after acid treatment; and lane 6, lysed cells.

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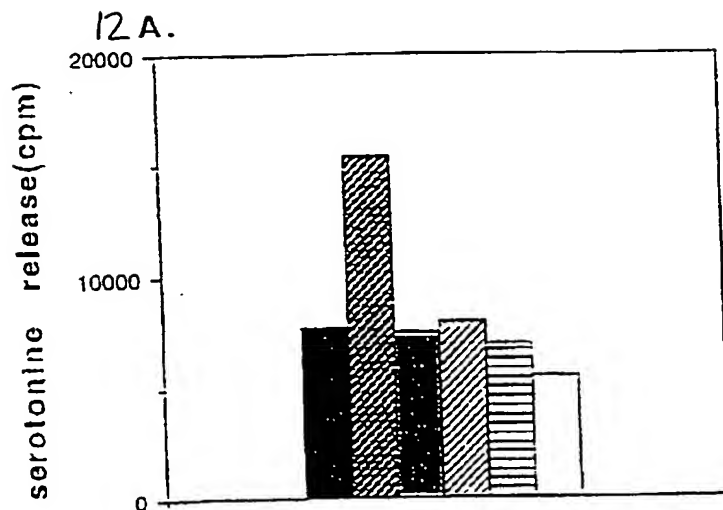


Figure 12A the effect of FC₂₋₃-PE40 on serotonin release from C57 cells. A) Cells were labeled overnight with [³H] hydroxytryptamine creatinine sulfate. The cells were then washed and exposed to various concentrations of FC₂₋₃-PE40 for 30 minutes. Control cells were pre-incubated with IgE and exposed to DNP and [³H] hydroxytryptamine creatinine sulfate released into the medium was measured:

■ control, ▨ IgE-DNP, ▩ 100ng, ▪ 250,
 ▫ 1000ng, or □ 5000 ng FC₂₋₃-PE40.

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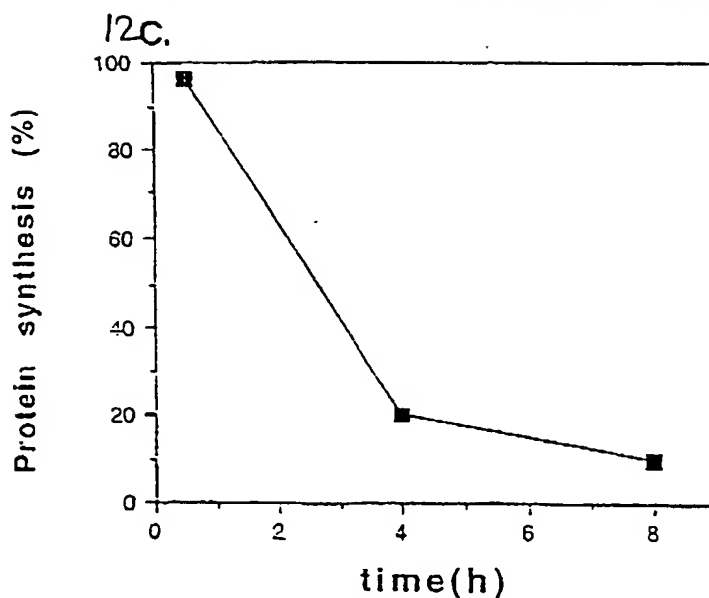
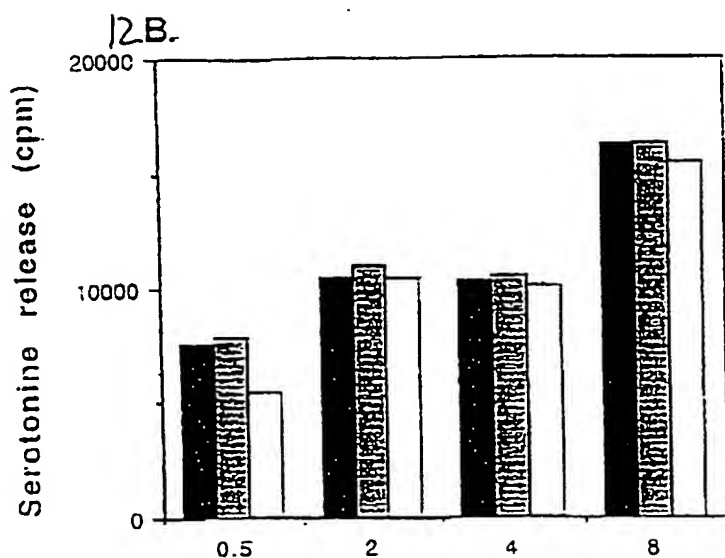


FIGURE 12 B) Cells were incubated with FC_{2-3} -PE40. At different time points $[^3H]$ hydroxytryptamine sulfate release into the medium was measured; legends as in FIGURE 12C) Time dependent cytotoxicity of FC_{2-3} -PE40 against C57 cells. Unlabeled cells were incubated as in (B). At the same time points cells were pulsed for 1 h with $[^3H]$ leucine and its incorporation into cellular proteins was measured. The results are expressed as the percentage of protein synthesis of control cells not exposed to chimeric proteins.

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Table 1: Cytotoxicity of Fc_{2-3} -PE₄₀ chimeric protein against various mouse cells

| | Cell line | Cell Origin | ID ₅₀ (ng/ml) |
|---------------------|-------------------|--|--------------------------|
| TARGET CELLS | MC-9 | Mast cells | 50-100 |
| | C57 | Mast cells | 100-125 |
| | BMMC | Primary bone marrow-derived mast cells | |
| | Abelson | Transformed mast cells | 1,200-1,500 |
| NON-TARGET CELLS | HEMOPOETIC | | |
| | L ₁₀ A | B cell, non-secreting | >10,000 |
| | X ₁₆ B | B cell, non-secreting | >10,000 |
| | UT | B cell, non-secreting | >10,000 |
| | PD1.1 | T cell, immature | >10,000 |
| | EL-4 | T cell, mature | >10,000 |
| | Erythro-leukemia | | >10,000 |
| | CONNECTIVE TISSUE | | |
| | L TK | Fibroblast | 1900 |
| | Hepatoma | | 1500 |

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 96/00181

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 //C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| Y | US 5 082 927 A (PASTAN IRA ET AL) 21 January 1992 see the whole document --- | 1-11 |
| Y | NATURE, vol. 331, 14 January 1988, pages 180-183, XP002026902 HELM ET AL.: "The mast cell binding site on human immunoglobulin E" cited in the application see the whole document --- | 1-11 |
| A | WO 91 11456 A (TANOX BIOSYSTEMS INC) 8 August 1991 see the whole document --- | 1-11 |
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Date of the actual completion of the international search

6 March 1997

Date of mailing of the international search report

07.04.97

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INTERNATIONAL SEARCH REPORT

International Application No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
| A | THE JOURNAL OF IMMUNOLOGY, vol. 140, no. 8, 15 April 1988, pages 2585-2588, XP002026903 KITANI ET AL.: "INHIBITION OF ALLERGIC REACTIONS WITH MONOCLONAL ANTIBODY TO THE HIGH AFFINITY IgE RECEPTOR" cited in the application see the whole document --- | 1-11 |
| A | WO 94 04689 A (US HEALTH) 3 March 1994 see the whole document --- | 1-11 |
| A | WO 90 12592 A (US ARMY ;PROTEIN DESIGN LAB INC (US)) 1 November 1990 see the whole document --- | 1-11 |
| A | NATURE, vol. 339, no. 6223, 1 June 1989, pages 394-397, XP000026214 CHAUDHARY V K ET AL: "A RECOMBINANT IMMUNOTOXIN CONSISTING OF TWO ANTIBODY VARIABLE DOMAINS FUSED TO PSEUDOMONAS EXOTOXIN" see the whole document --- | 1-11 |
| A | THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 19, 5 July 1988, pages 9470-9475, XP002026904 KONDO ET AL.: "ACTIVITY OF IMMUNOTOXINS CONSTRUCTED WITH MODIFIED PSEUDOMONAS EXOTOXIN A LACKING THE CELL RECOGNITION DOMAIN" see the whole document ----- | 1-11 |

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. Application No

PCT/IL 96/00181

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| US 5082927 A | 21-01-92 | US 4892827 A | 09-01-90 |
| | | AU 618722 B | 09-01-92 |
| | | AU 8021187 A | 21-04-88 |
| | | CA 1336691 A | 15-08-95 |
| | | DE 3789587 D | 19-05-94 |
| | | DE 3789587 T | 28-07-94 |
| | | EP 0261671 A | 30-03-88 |
| | | EP 0583794 A | 23-02-94 |
| | | ES 2074042 T | 01-09-95 |
| | | IE 66223 B | 13-12-95 |
| | | IL 83971 A | 24-06-94 |
| | | JP 2502063 T | 12-07-90 |
| | | NO 180270 B | 09-12-96 |
| | | WO 8802401 A | 07-04-88 |
| ----- | | | |
| WO 9111456 A | 08-08-91 | US 5260416 A | 09-11-93 |
| | | US 5274075 A | 28-12-93 |
| | | AU 5214893 A | 03-03-94 |
| | | AU 645783 B | 27-01-94 |
| | | AU 7317991 A | 21-08-91 |
| | | CA 2074089 A | 24-07-91 |
| | | EP 0512064 A | 11-11-92 |
| | | JP 5504482 T | 15-07-93 |
| | | US 5342924 A | 30-08-94 |
| | | US 5514776 A | 07-05-96 |
| | | US 5254671 A | 19-10-93 |
| ----- | | | |
| WO 9404689 A | 03-03-94 | AU 5098393 A | 15-03-94 |
| ----- | | | |
| WO 9012592 A | 01-11-90 | AT 138688 T | 15-06-96 |
| | | AU 641392 B | 23-09-93 |
| | | AU 5562790 A | 16-11-90 |
| | | CA 2053911 A | 22-10-90 |
| | | DE 69027210 D | 04-07-96 |
| | | DE 69027210 T | 23-01-97 |
| | | EP 0469065 A | 05-02-92 |
| | | ES 2091824 T | 16-11-96 |
| | | JP 8029101 B | 27-03-96 |
| | | JP 4504363 T | 06-08-92 |
| ----- | | | |

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